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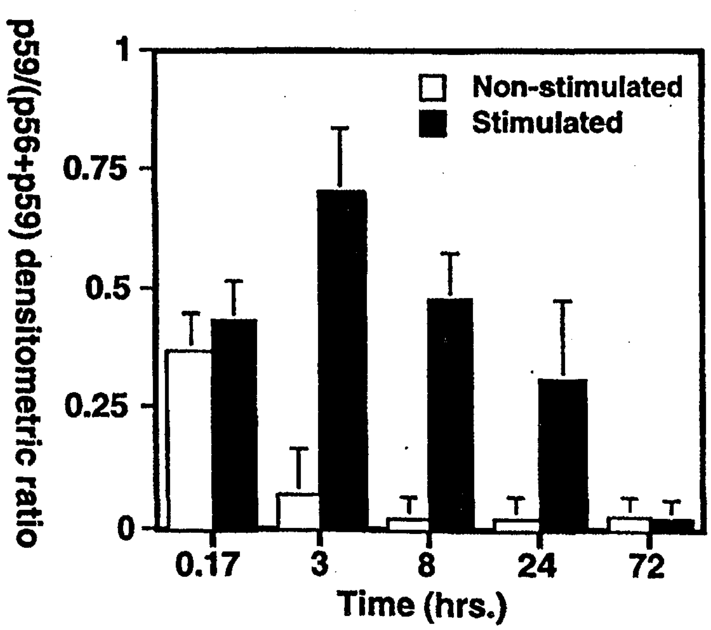
(57) Abstract

The invention features an assay which allows the correlation between the ratio of serine phosphorylated lck to total lck with the level of T cell activation. This assay may be used for the rapid determination of the efficacies of therapies which either induce or prevent T cell activation. Also provided are src-family tyrosine kinase polypeptides which have reduced serine phosphorylation-mediated degradation.



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LCK SERINE PHOSPHORYLATION ASSAY FOR T-CELL ACTIVATION AND ACTIVATION-INDUCED CELL DEATH

Background of the Invention

The field of this invention is assays used to identify T cell activation and modulation of ensuing activation-induced T cell death.

The cellular and humoral branches of the antigen-specific immune response are initiated by activated thymus-derived T lymphocytes (T cells). The signal from the T cell receptor (TCR) upon recognition of specific complexes of peptide with major histocompatibility complex (MHC) molecules, simultaneously delivered with signals from multiple accessory molecules upon engagement with their cognate ligands on the antigen presenting cell (APC), will initiate antigen-specific T cell activation. The first T cell activation event to be detected, within seconds of TCR engagement, is the rapid tyrosine phosphorylation of the CD3-γ, CD3-δ, CD3-ε, and TCR-ζ chains associated with the TCR. None of the chains associated with the T cell receptor, however, bears intrinsic protein tyrosine kinase activity.

One of the members of the src-family of protein tyrosine kinases, lck, has been shown to play an important role in TCR mediated signal transduction. This membrane-localized kinase is associated with cytoplasmic domains of the CD4 or CD8 coreceptors for the MHC class II or class I molecules, found on helper or cytotoxic T cells, respectively. When the TCR is ligated with antigenic peptide plus MHC on an APC, CD4 or CD8 is recruited to the TCR, bringing lck into the vicinity of the receptor and its associated chains, leading to the rapid formation of the tyrosine phosphorylated TCR signaling complex, and, ultimately, to the changes in gene expression that characterize T cell activation.

To achieve antigen-induced activation, the T cell must be resting prior to the

engagement of the TCR. Should the cell be cycling when the TCR is engaged, the T cell will die, an event which is characteristic of a phenomenon known as activation-induced cell death. However, a very early step in T cell activation is the proliferation of the cells. Frequently, the antigen is present for a considerable length of time during this proliferation. The mechanisms by which newly cycling stimulated T cells evade death normally induced by the still-present antigen are poorly understood. A better understanding of how cell death is avoided in this context would facilitate the identification of compounds which either generate or prevent T cell activation. In the field of clinical medicine, methods which affect T cell activation following a prolonged TCR engagement will be highly useful in, for example, enhancing rejection of tumor cells or facilitating acceptance of allogeneic or xenogeneic organ transplants.

Summary of the Invention

I have discovered a simple and rapid method for detecting increased T cell activation by measuring the levels of 59 kilodalton (kDa) lck and total lck. Since activated T cells are the initiators of antigen-specific immunity, this method will allow the rapid detection of an antigen-specific immune response. This method will also be useful in screening candidate therapeutic compounds and protocols for their efficacy in either stimulating or blocking the antigen-specific immune response. Identification and development of such compounds and protocols is useful for enhancing, decreasing, or preventing antigen-specific immune responses. Therapies which enhance the immune response aid in the development of immunity to antigens derived from, among others, opportunistic pathogens and cancerous cells. Therapies which prevent or decrease the development of an antigen-specific immune response are useful in preventing an immune response to antigens derived from, among others, allogeneic or xenogeneic organ transplants.

The method for detecting increased T cell activation of the present invention is

based upon my discovery that the generation of a T cell activation response to a prolonged engagement of the TCR correlates with decreased TCR-mediated signaling and an increasing ratio of lck with an apparent molecular mass of 59 kilodaltons (kDa) to total lck.

As an extension of the above finding, I believe that if the activation-induced serine phosphorylation of lck is prevented, T cell activation-induced cell death will occur. Hence, the invention described herein also provides methods to prevent serine phosphorylation-mediated degradation and thereby prevent T cell activation by allowing T cell activation-induced cell death to occur.

In a first aspect, the invention features a method for detecting the level of T cell activation that includes the steps of: (a) providing a first sample that includes a T cell; (b) measuring the amount of serine phosphorylated lck in the first sample; and (c) measuring the amount of total lck in the first sample, where an increase in the ratio of serine phosphorylated lck to total lck relative to the ratio of serine phosphorylated lck to total lck in a second sample consisting essentially of unstimulated T cells indicates increased T cell activation in the first sample, and where a decrease in the ratio of serine phosphorylated lck to total lck relative to the ratio of serine phosphorylated lck to total lck in the second sample indicates increased T cell activation-induced cell death in the first sample. In one embodiment of this aspect of the invention, the first sample and the second sample are from two different individuals. In another embodiment of this aspect of the invention, first sample and the second sample are from the same individual, and the second sample is collected from the individual prior to the collection of the first sample. In another embodiment, the T cell of the first sample and the unstimulated T cells of the second sample are $CD4^+$ helper T cells. In another embodiment, the T cell of the first sample is a stimulated cell, *e.g.*, stimulated with a peptide-MHC complex. In another embodiment, the T cell may be stimulated

with a peptide-MHC complex expressed on an antigen presenting cell (*e.g.*, a cell that also expresses ICAM-1 and/or CD80). Preferably, the T cell used in the methods of the invention is stimulated for at least one hour, optimally 3 hours, and is stimulated for less than 24 hours.

In another embodiment of the first aspect of the invention, the method includes detecting the level of T cell activation T cell activation in a cell-free system or a modified cell system. In related embodiments, the lck may be a recombinant protein or the T cell activation response may be initiated by components which mimic the T cell receptor. In a preferred embodiment, the method of the first aspect of the invention is used to identify a compound that alters T cell activation.

In a second aspect, the invention features a method for detecting a compound that modulates T cell activation. This method includes the steps of: (a) providing a first sample that includes a T cell; (b) contacting the first sample with a compound; (c) measuring the amount of the serine phosphorylated lck in the compound-contacted first sample; and (d) measuring the amount of total lck in the compound-contacted first sample, where a change in the ratio of the serine phosphorylated lck to total lck in the compound-contacted first sample, relative to the serine phosphorylated lck to total lck ratio in a second sample that includes a T cell, where the second sample is not contacted with the compound, indicates that the compound modulates T cell activation. In one embodiment of this aspect of the invention, the T cell of the first sample and the T cell of the second sample is a stimulated T cell.

In another embodiment of the second aspect of the invention, the compound that modulates T cell activation is a compound that increases T cell activation if the change is at least 20% higher than the ratio of the second sample. Preferably, the change is optimally 60% higher as compared to the ratio of the second sample for a compound that increases T cell activation.

In another embodiment of the second aspect of the invention, a compound that

modulates T cell activation is a compound that increases T cell activation-induced cell death if the amount of the change is at least 50% lower than the ratio of the second sample.

In a third aspect, the invention features a kit which includes an antibody capable of specifically binding lck, and mean for determining the apparent molecular mass of lck (*e.g.*, a size-differentiating immunoassay, *e.g.*, Western blotting analysis). Kits which include antibodies which specifically recognize all lck with or without post-translational modification are part of the kits of the invention.

In a fourth aspect, the invention features a src-family tyrosine kinase polypeptide having a mutation that reduces serine phosphorylation-mediated degradation, where the mutation is a serine to alanine mutation of the serine residue located at the amino terminus of a sequence selected from SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, or SEQ ID NO: 25. In one embodiment of this aspect of the invention, the polypeptide is lck (*e.g.*, from a mouse or human).

In a fifth aspect, the invention features a src-family tyrosine kinase polypeptide having a mutation that reduces serine phosphorylation-mediated degradation, where the mutation is a leucine-leucine to alanine-alanine mutation of the leucine-leucine residues located in a sequence selected from SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, or SEQ ID NO: 25. In one embodiment of this aspect of the invention, the polypeptide is lck (*e.g.*, from a mouse or human).

In a sixth aspect, the invention features a method for generating a src-family tyrosine kinase polypeptide that has a reduced level of serine phosphorylation-mediated degradation that includes the steps of: (a) identifying SEQ ID NO: 27 or SEQ ID NO: 28 in the polypeptide; and (b) mutating the 5' serine residue in the SEQ ID NO: 27 or the SEQ ID NO: 28 to alanine, where the mutation reduces the level of the serine phosphorylation-mediated degradation in the polypeptide.

The invention also provides methods utilizing lck polypeptides and fragments thereof which are obtained from all living organisms found to synthesize lck proteins. Furthermore, in addition to using peptide fragments as antigens, the invention also provides methods of using substantially full-length polypeptides as antigens to activate T cells. The invention also provides for methods which engage the T cell receptor, other than peptide plus MHC molecules complexes, such as antibodies which bind T cell receptor associated chains, or bacterially derived superantigens.

For the purposes of the present invention the following terms are defined below.

"Antigen presenting cell (APC)" means a cell capable presenting an antigen in such a way that it can stimulate an immune response. This definition is not limited to a "classical APC" (such as a macrophage), but is expanded to include cells which can serve as antigen presenting cells by an ability to express the appropriate cell surface molecules (e.g., MHC). Preferably, the APC constitutively expresses major histocompatibility complex (MHC) molecules at its cell surface. Preferably, an APC expresses the ICAM-1 cell surface molecule or the CD80 cell surface molecule. More preferably, an APC expresses both the ICAM-1 and CD80 cell surface molecules.

"Antigen-specific" means an immune response specific toward one antigen.

"Helper T cell" means a thymus-derived lymphocyte capable of generating helper T cell responses usually expressing the CD4 cell surface molecule.

"Cytotoxic T cell" means a thymus-derived lymphocyte capable of cytotoxic

function usually expressing the CD8 cell surface molecule.

"Total lck" means all lck protein with forms of various apparent molecular masses due to post-translational modification.

"Serine phosphorylated lck" means the serine phosphorylated lck protein with an apparent molecular mass of 59-60 kilodaltons.

"MHC" means the major histocompatibility complex (MHC) molecules.

"Peptide-MHC" means peptide in association with MHC molecules.

"Recombinant protein" means a protein that is synthesized by a method which includes a step or reagent requiring human or mechanical intervention or input.

"T cell receptor (TCR) desensitization" means loss of signaling capability upon persistent engagement with agonist ligand.

"Serine phosphorylation-mediated degradation" is meant the phosphorylation on one or more serine residues in a protein which causes the serine phosphorylated protein to undergo a more rapid degradation than is observed in the same protein that is not serine phosphorylated.

"Protein" or "polypeptide" means any chain of amino acids, regardless of length or post-translational modification (for example, glycosylation or phosphorylation).

"Peptide" or "peptide fragment," as applied to a protein or polypeptide, will ordinarily be less than 50 contiguous amino acids, most preferrably about 9 to 25 contiguous amino acids in length.

"Antibody that specifically binds lck" means an antibody that recognizes and binds the lck protein or polypeptide fragments thereof but that does not substantially recognize and bind other molecules in a sample, *e.g.*, a biological sample, that naturally includes protein. Preferably, an antibody that specifically binds lck will bind an lck protein or fragment thereof with a dissociation constant (K_D) of at least 10^{-7} under standard immunodetection conditions.

Brief Description of the Drawings

Fig. 1 is a Western blot analysis of tyrosine phosphoprotein expression levels in stimulated 3C6 cells.

Fig. 2 is a Western blot analysis of tyrosine phosphorylation levels of CD3-ε, TCR-ζ, and ZAP-70 in CD3-ε immunoprecipitations of stimulated 3C6 cells.

Fig. 3 is a graph illustrating 3C6 T cell proliferation induced by stimulation.

Fig. 4 is a flow cytometric profile of T cell receptor expression on 3C6 cells.

Fig. 5 is a graph illustrating the levels of TCR and CD3-ε expression on stimulated 3C6 cells.

Fig. 6 is a graph illustrating cell death percentages of stimulated and unstimulated 3C6 cells.

Fig. 7 are flow cytometric profiles of TCR and CD4 expression on stimulated 3C6 cells.

Fig. 8 is a Western blot analysis of lck levels in stimulated 3C6 cells.

Fig. 9 is a graph of the ratio of 59 kDa lck to total lck from stimulated 3C6 cells.

Fig. 10 is a Western blot analysis of lck levels in CD4 immunprecipitates prepared from unstimulated and stimulated 3C6 cells.

Fig. 11 is an *in vitro* kinase assay of lck immunoprecipitated from stimulated 3C6 cells.

Fig. 12 is a graph of the quantitation of lck kinase activity from stimulated 3C6 cells.

Fig. 13 is a series of photographs showing the intracellular localization of lck-GFP (lck:pEGFP) and CD4-lck-GFP (CD4:lck:pEGFP) in transfected BHK-21 cells following 0, 15, 30, 60, and 90 minutes of stimulation with 12.5 μg/ml PMA. (Intracellular Localization of *lck* Chimeras in BHK-21 Transfectants Upon PMA

Treatment. All transfected BHK-21 cells stimulated with 12.5 μg/ml PMA. Time course indicated as minutes post-stimulation.)

Fig. 14 is a schematic diagram indicating the Ser-Xaa_{7-8}-Leu-Leu (Xaa is any amino acid residue) motifs found in various src-family kinases, with murine lck being

the model src-family tyrosine kinase.

Detailed Description of the Invention

The invention provides a simple and efficient method for identifying agents capable of initiating or preventing T cell activation following a prolonged engagement of the T cell receptor. Antigen-induced activation of T cells is an initiating event in the generation of antigen-specific immunity, hence identification of agents which affect T cell activation will aid in the development of therapies which can either stimulate or block the T cell mediated antigen-specific immune response. T cell activation inducing therapies will be useful in stimulating an immune response to opportunistic pathogens, cancerous cells, and other disease causing agents, whereas therapies which decrease or prevent T cell activation will be useful in preventing an immune response to antigens derived from, among others, allogeneic or xenogeneic organ transplants.

Although a cycling T cell will die when its TCR is engaged with antigenic peptide-MHC, a newly activated T cell entering the cell cycle is able to escape this activation-induced cell death. Using tyrosine phosphorylation of TCR associated chains as a marker for T cell activation, I observed that maximal tyrosine phosphorylation induction occurred ten minutes following engagement of the TCR, and that this induction level had decreased following a one hour engagement of the TCR, and remained low up to a 24 hour engagement of the TCR. I termed this diminished ability of the engaged TCR to induce tyrosine phosphorylation "TCR desensitization." TCR desensitization due to persistent receptor engagement, however, had no effect on activation-induced proliferation, indicating that TCR desensitization is a normal event in antigen-induced T cell activation.

Activation of $CD4^+$ T cells results in co-modulation of the TCR and the co-receptor molecule, CD4. The TCR is rapidly recycled back to the cell surface without

the need for new protein synthesis, unlike CD4, which is degraded upon internalization. I discovered that prolonged antigen-induced T cell activation leads to a state of dissociated cell surface expression of TCR and CD4, and that this dissociated expression of TCR and CD4 parallels the time frame of TCR desensitization. Hence, the appearance of $CD4^-$ cells in the population is another marker for TCR desensitization and, correspondingly, antigen-specific T cell activation.

Since lck is associated with the cytoplasmic domain of CD4, I next looked at the effects of TCR desensitization upon lck. Following prolonged engagement of the TCR with peptide-MHC, I observed a change in the ratio of two electrophoretically dissimilar forms of lck. I noticed a decrease in what is normally the most abundant form of lck, *i.e.*, the form having an apparent molecular mass of 56 kilodaltons (kDa) after a one hour exposure of the T cells to peptide-MHC. In contrast, the relative amount of the lck form with an apparent molecular mass of 59 kDa, which corresponds to a serine-phosphorylated form of lck, increased from 80 to 100% higher than the amount of 59 kDa observed in unstimulated T cells this same time frame. As the exposure time of the T cells to peptide-MHC increased past 1 hour, the amount of total lck continued to decrease to 90-100% lower than the amount observed in unstimulated T cells, as did the level of lck kinase activity, indicating that this serine phosphorylated form of lck may be catalytically inactivated. Hence, I discovered that a simple method for detecting TCR desensitization, and, thereby, detecting the generation of antigen-specific T cell activation, was to observe an increase in the amount of the 59 kDa serine phosphorylated form of lck tyrosine kinase relative to total lck following a greater than 10 minute engagement of the TCR. This same observation is seen in cells stimulated via non-TCR pathways (e.g., PKC activation with phorbol ester).

The following examples are to illustrate the invention. They are not meant to

limit the invention in any way.

Example I

Antigen-induced, TCR-mediated tyrosine phosphorylation of T cell lysates decreases with persistent engagement with peptide-MHC molecule complexes.

Methods

1×10^6 P13.9 murine fibroblast cells which express the murine MHC class II molecules, I-E^k, as well as costimulatory molecules ICAM-1 and CD80 (Madrenas *et al.*, Science 267: 515-518, 1995; Ronchese *et al.*, Nature, 329: 254-256, 1987; Konig, *et al.*, J. Exp. Med., 182: 779-787, 1995), were intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) to stimulate 1×10^6 3C6, a murine $CD4^+$ helper T cell clone specific for pigeon cytochrome C fragment 81-104 (PCC(81-104) peptide) bound to I-E^k peptide (Matis *et al.*, J. Immunol. 130: 1527-1535, 1983) for 10 minutes, 3 hours, 8 hours, or 24 hours, in the presence of either no peptide, or 1 μM, 10 μM, or 100 μM of commercially available purified PCC(81-104) peptide (sequence 5' IFAGIKKKAERADLIAYLKQATAK 3'; SEQ ID NO: 1), in 2 mls total culture volume. 3C6 T cells were then isolated from the co-culture by magnetic depletion of the fibroblasts (Madrenas *et al.*, Proc. Natl. Acad. Sci. USA 93: 9736-9741, 1996). For induction of tyrosine phosphoprotein studies, the 3C6 T cells were immediately lysed, with the lysate (6×10^5 T cell equivalents) or CD3-ε immunoprecipitates (using commercially available antibody) of the lysate (9×10^5 T cell equivalents) SDS-PAGE resolved and immunoblotted with a commercially available anti-phosphotyrosine antibody as described in Madrenas *et al.*, Science 267: 515-518, 1995. For proliferation studies, the 3C6 cells stimulated with mitomycin C treated P13.9 cells with either no peptide or 10 μM purified PCC(81-104) peptide for 30 minutes, 1 hour, 3 hours, 8 hours, 24 hours, and 48 hours, were returned to culture for 48 hours total, with the addition of 3H-thymidine to the cells in the final 20 hours

of culture. The cells were then lysed, and proliferation was measured by amount of ^{3}H-thymidine uptake, as described in Madrenas *et al.*, Science 267: 515-518, 1995.

Results

Stimulation of T cells with immunogenic peptide:MHC molecule complexes on APC for 10 minutes induces tyrosine phosphorylation of TCR subunits (CD3 and TCR ζ chains) and of multiple downstream proteins. Immunoblot analysis of tyrosine phosphorylation of 3C6 T cell lysates after a 10 minute stimulation with PCC(81-104) peptide plus P13.9 cells showed the appearance of newly tyrosine phosphorylated proteins or an increase in tyrosine phosphorylation for proteins in the 36 to 40, 50-60, 70, 80, 90, 120, 140, and 150 kDa ranges, as is shown on Fig. 1. However, when the 3C6 T cells were stimulated for longer, I saw a progressive decrease in peptide-induced tyrosine phosphorylation of T cell lysates. As is shown on Fig. 1, this decrease was already apparent by 3 hours and was readily evident by 8 and 24 hours. Loss of tyrosine phosphorylation with persistent T cell stimulation correlated with the magnitude of the initial increase of tyrosine phosphorylation with proportionally greater loss with higher concentrations of peptide. The same kinetics of cessation of signaling after initial activation were seen with different peptide concentrations.

I next examined whether a decrease in tyrosine phosphorylation of the chains associated with the T cell receptor (TCR) correlated with decreased TCR-mediated signaling. Tyrosine phosphorylation of the TCR ζ and CD3-ε chains in CD3-ε immunoprecipitates progressively decreased with increased time of exposure of 3C6 cells to PCC(81-104) peptide plus P13.9 cells, as depicted on Fig. 2. The kinetics and dose dependence of the loss of TCR-mediated signaling with persistent stimulation was parallel to the loss in tyrosine phosphorylation of T cell lysates, being already observed at 3 hours and maximal between 8 and 24 hours of antigen stimulation. I further observed that the loss of TCR subunit tyrosine phosphorylation started to reverse after 24 hours, and reversal was complete by 48 to 72 hours. These results

demonstrate that persistent exposure to immunogenic peptide:MHC molecule complexes on APC lead to a loss of TCR-mediated signaling.

Next, I examined the relationship between the decrease of TCR-mediated signaling upon persistent receptor engagement and proliferation, a downstream event caused by T cell activation. 3C6 T cells were stimulated with PCC (81-104) peptide plus P13.9 cells for different times, after which T cells were harvested by magnetic depletion of APC and cultured for the remaining 48 hours, with proliferation measured by 3H-thymidine uptake in the last 20 hours of culture. The result of this experiment, shown on Fig. 3, demonstrated that antigen-specific T cell proliferation was slightly but significantly elevated after just 30 minutes of antigen stimulation and increased with time up to 24 hours. However, when PCC (81-104) peptide plus P13.9 cells were present during the complete 48 hour culture, T cell proliferation was significantly reduced. This result indicates that the progressive loss of TCR-mediated signaling upon persistent antigen stimulation did not interfere with T cell commitment and proliferation if the antigen is not present for longer than 24 hours. Since the increase in T cell proliferation seen with longer time exposure to APC may be due to activation of more T cells, then these results indicate that the loss of TCR-mediated signaling may protect T cells from activation-induced cell death during the first 24 hours following activation.

Example II

The decrease in TCR-mediated signaling is not due to lack of antigen presentation, loss of TCR expression, TCR subunit dissociation, or T cell death.

Methods

P13.9 cells were intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) to stimulate 3C6 cells for varying amounts of time in

the presence of varying concentrations of purified PCC(81-104) peptide. For restimulation studies, the T cells were then isolated by magnetic depletion of the fibroblasts and immediately returned to co-culture for 10 minutes with fresh APC in the presence of purified PCC(81-104) peptide, re-isolated, and immediately lysed, with the lysate (6 x 10^5 T cell equivalents) SDS-PAGE resolved and immunoblotted with a commercially available anti-phosphotyrosine antibody. For cell surface expression studies, 3C6 cells isolated following a 3 hour stimulation in 1 ml total culture volume with APC with either no peptide or 10 μM peptide were cell surface stained with a primary antibodies directed against either the TCR (Kubo *et al.*, J. Immunol. 142: 2736-2742, 1989) or CD3-ε (commercially available) followed by a fluorescein-labeled secondary antibody, and analyzed by flow cytometry on a Becton-Dickinson® FACScan. For cell death studies, 3C6 cells isolated following a 24 hour stimulation with APC with either no peptide or 10 μM peptide were stained with Trypan blue, a dye which stains dead but not living cells. Viability of T cells was measured in triplicate by Trypan blue exclusion.

Results

The following potential causes of decreased TCR-mediated signaling were excluded. First, the decrease in TCR-mediated tyrosine phoshorylation with time was not due to lack of PCC(81-104) peptide presentation secondary to either death of APC or to peptide degradation because restimulation with fresh peptide at the same initial concentration and APC for 10 minutes did not restore the tyrosine phosphorylation response of those T cells. Second, the time-dependent decrease in T cell responsiveness was not a consequence of lack of TCR expression following activation of T cells and TCR internalization. As shown on Fig. 4, the expression of TCR decreased slightly after 3 hours of stimulation (up to 35% in some experiments), but was restored consequently to levels similar to those on non-stimulated T cells. This is in contrast to TCR signaling that was progressively reduced from 3 to 24 hours, as

seen on Fig. 1. Third, as demonstrated on Fig. 5, the loss of antigen-induced TCR-mediated signaling was not due to lack of CD3-ε (or dissociated expression of TCR and CD3-ε on the membrane because expression of CD3-ε paralleled expression of TCR αβ complex. Fourth, the loss of TCR-mediated signaling by 24 hours of T cell stimulation was not due to activation-induced cell death because the results in Fig. 6 demonstrate no significant antigen-induced increase in cell death was observed by Trypan blue exclusion method.

Thus, these experiments show that the ability of the TCR to signal is lost during persistent stimulation with peptide:MHC molecules on APC and cannot be recovered by addition of fresh peptide and APC. I term this phenomenon TCR desensitization

Example III

TCR desensitization occurs with functional TCR-CD4 dissociation.

Methods

P13.9 cells were intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) to stimulate 3C6 cells in the presence of 10 μM purified PCC(81-104) peptide for 0, 3, 8, and 24 hours. T cells were then isolated from the co-culture by magnetic depletion of the fibroblasts, and were either cell surface stained with primary antibodies directed against the T cell receptor (Kubo *et al.*, J. Immunol. 142: 2736-2742, 1989) or against CD4 (Dialynas *et al.*, J. Immunol. 131: 2445-2451, 1983), followed by appropriate secondary staining with fluorescein or phycoerythrin-labeled antibody and analyzed by flow cytometry on a Becton-Dickinson® FACScan.

Results

Activation of T cells through the TCR causes co-modulation of CD4 co-receptor molecules. However, TCR and CD4 are differentially targeted after internalization. The TCR is recycled back to the cell surface as indicated by its re-

expression being non-sensitive to protein synthesis inhibitors. In contrast, CD4 is targeted to a lysosomal compartment and degraded; as a result, re-expression of CD4 requires new protein synthesis. Given this difference in internalization outcomes, I hypothesized that antigen-induced T cell activation could lead to a state of "dissociated"cell surface expression of TCR and CD4 due to a delay in re-expression of CD4 upon antigen-induced T cell activation. This could correlate with TCR desensitization given the critical roles that CD4 plays in stabilization of the TCR:peptide:MHC molecule complex interaction and in the establishment of the early TCR signaling patterns through its non-covalent association with the src-family protein tyrosine kinase, lck. Therefore, I looked at the stimulation-induced changes in membrane expression of TCR and CD4 molecules within the time frame of TCR desensitization. I confirmed that, after an initial decrease in surface levels and as early as 8 hours after T cell activation, the TCR was normally re-expressed. However, as depicted on Fig. 7, CD4 expression decreased steadily to a plateau of 20% of the original level. As a result, there was a dissociated expression of TCR and CD4 that paralleled the time frame of the decrease in TCR-mediated signaling, and that translated into the appearance of T cells that express TCR but not CD4 by 3 hours of stimulation and an increase in the proportion of these cells during the 24 hour stimulation.

Example IV

TCR desensitization correlates with serine phosphorylation of lck and inactivation and loss of lck.

Methods

P13.9 cells were intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) to stimulate 3C6 cells in the presence of either no peptide or 10 μM purified PCC(81-104) peptide for 0 minutes, 1 minute, 10 minutes, 1

hour, 3 hours, 8 hours, 24 hours, and 72 hours. T cells were then isolated from the co-culture by magnetic depletion of the fibroblasts. For lck mobilization and quantitation studies, the T cells were immediately lysed, and lysates (6×10^5 T cell) were SDS-PAGE resolved and immunoblotted with a commercially available anti-lck antibody. For studies of lck associated with CD4, CD4 immunoprecipitates were prepared as described in Dialynas *et al.*, J. of Immunol. 131: 2445-2451, 1983, were SDS-PAGE resolved and immunoblotted with a commercially available anti-lck antibody. Relative amounts of lck were quantitated using an imaging densitometer (Bio-Rad, model GS 700, Hercules, CA) and the Molecular Analyst® Software (version 1.0, 1994, Bio-Rad laboratories). For lck kinase activity studies, lck immunoprecipitates prepared as described in Wiest *et al.* (J. Exp. Med. 178: 1701-1712, 1993) from the lysate (9×10^5 T cell equivalents) were incubated in 20 mM HEPES, pH 7.5, 100 mM NaCl, 5 mM $MnCl_2$, 2 mM NaATP, 10 μg/sample enolase, and 10 μCi 32γ-ATP for 5 minutes at room temperature, SDS-PAGE resolved, and analyzed using a phosphorimager.

Results

The biochemical effects of expression of TCR without available CD4, induced by persistent antigen-stimulation, have not been previously examined. However, this is of interest considering this "dissociated" expression may limit the amount of lck available for TCR-mediated signaling in a CD4-dependent response, and be subsequently responsible for the loss of TCR-mediated signaling. Therefore, I examined how lck is regulated during TCR desensitization. First, I looked at changes in levels of lck by Western blotting of cell lysates from antigen-stimulated T cells. As shown in Fig. 8, two different forms of lck were detected in these blots: the 56 kDa "basal" form and the 59 kD form that appears as a consequence of serine phosphorylation of lck (Veillette *et al.*, Mol. Cell. Biol. 8: 4353-4361, 1988; Veillette *et al.*, Oncogene Res. 2: 385-401, 1988; Marth *et al.*, J. Immunol. 142: 2430-2437,

1989; Watts *et al.*, J. Biol. Chem. 268: 23275-23282, 1993; Winkler *et al.*, Proc. Natl. Acad. Sci. USA 90: 5176-5180, 1993; and Oetken *et al.*, Mol. Immunol. 31: 1295-1302, 1994). The amount of 56 kDa lck did not significantly change during 24 hours of T cell-APC co-culture in the absence of peptide. However, the levels of this form decreased with time upon stimulation with PCC(81-104) peptide and APC. Simultaneously, the levels of serine-phosphorylated 59 kDa lck increased upon persistent stimulation in a parallel way to the loss of 56 kDa lck. These changes were associated with a progressive loss of total lck, maximal by 8-24 hours after which the levels recovered back to initial levels by 48 hours.

To correct the changes in the levels of each lck form for the loss of both forms of lck ("total" lck), I determined the 59 kDa:(56 kDa + 59 kDa) ratio as an indicator of the relation between the levels of shifted 59 kDa lck and the total amount of lck. As shown on Fig. 9, this ratio was significantly higher for stimulated T cells during the 24 hour stimulation. However, it peaked after 3 hours of stimulation decreasing afterwards, as the total lck levels decreased, and returned to basal levels by 72 hours. Thus, these results demonstrate that persistent T cell stimulation was associated with a shift from the 56 kDa lck to the 59 kDa serine-phosphorylated lck, and a subsequent loss in the total levels of lck. This decrease is not due to redistribution of lck to the cytoskeleton, as reported for HIV gp120-induced TCR desensitization, because I observed that the levels of this kinase in the "detergent-insoluble" fraction ("cytoskeletal" lck) were not significantly changed with persistent stimulation with peptide and APC. In addition, as demonstrated in Fig. 10, similar changes to those shown for total lck were also seen when CD4-associated lck levels were examined.

Next, I examined if the modifications of lck correlated with a loss of lck kinase activity. T cells were stimulated with PCC(81-104) and APC for different times. T cells were then harvested and lysed, followed by immunoprecipitation of lck and in vitro assay of the kinase activity of these immunoprecipitates. As demonstrated in

Fig. 11, with the activity quantitated by phosphorimager in Fig. 12, the kinase activity of lck, as measured by autophosphorylation of lck and/or phosphorylation of enolase, correlated with the changes seen in gel mobility and remained low for 24 hours. The decrease in kinase activity did correlate with a shift from 56 kDa lck to serine phosphorylated 59 kDa lck. Therefore, these results imply that generation of the serine phosphorylated 59 kDa lck is associated with a loss in kinase activity which, in turn, results in decreased TCR signaling following prolonged engagement of the TCR which, in turn, is indicative of T cell activation.

Hence, lck serine phosphorylation provides a simple method by which T cells which escape activation-induced cell death and achieve activation status following prolonged engagement of the TCR can be detected. If there is an increased amount of serine phosphorylated 59 kDa lck relative to the amount of total lck in stimulated T cells, this is a clear indication that the cells have escaped cell death and will achieved an increased activation status.

EXAMPLE V

Pervanadate stimulation of T cell activation and lck serine phosphorylation.

Methods

P13.9 cells were intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) in the presence of 100 μM purified PCC(81-104) peptide to stimulate 3C6 T cells for 10 minutes. The T cells are then isolated from the co-culture by magnetic depletion of the fibroblasts and 1 x 10^6 cells resuspended in 100 μl total volume. Each 100 μl sample was then either not treated, or treated with 0.55 ul or 0.07 μl of a 1 ml mixture containing 10 M $NaVO_4$ and 3.3 μl of 30% H_2O_2. The cells were then lysed, and the lysate (6 x 10^5 T cell equivalents) was SDS-PAGE resolved and immunoblotted with anti-phosphotyrosine antibody. Relative amounts of tyrosine phosphoproteins were quantitated on a densitometer. SDS-PAGE resolved

lysates are also immunoblotted with anti-lck antibody and quantitated on a densitometer.

Results

Peptide plus P13.9 stimulated 3C6 cells were found to induce a 50% higher amount of tyrosine phosphorylated proteins than unstimulated 3C6 cells following a 10 minute stimulation. Unstimulated pervanadate treated 3C6 cells did not induce tyrosine phosphorylated proteins. The stimulated pervanadate treated 3C6 cells, however, induced a 20% higher amount of tyrosine phosphorylated proteins than the stimulated, untreated 3C6 cells. Furthermore, a ratio of 59 kDa lck to total lck in pervanadate treated 3C6 cells is at least 20 percent, and preferably 60% higher than the ratio of 59 kDa lck to total lck in stimulated cells not treated with pervanadate. Hence, I was able to detect the T cell activation enhancing abilities of sub-stimulatory levels of pervanadate.

EXAMPLE VI

Testing ability of a compound to stimulate T cell activation.

Methods

P13.9 cells are intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) in the presence of 10 μM purified PCC(81-104) peptide for various amounts of time to stimulate 3C6 cells. The 3C6 cells are either not treated or previously, concurrently, or soon to be treated with a compound being tested for an ability to inhibit T cell activation. T cells are then isolated from the co-culture by magnetic depletion of the fibroblasts and immediately lysed. The lysate (6×10^5 T cell equivalents) is SDS-PAGE resolved and immunoblotted with an anti-lck antibody. Relative amounts of lck are quantitated on a densitometer.

Results

Peptide plus P13.9 stimulated untreated 3C6 cells are found to produce a higher

ratio of 59 kDa lck to total lck than unstimulated untreated 3C6 cells following stimulation for 1 to 24 hours, preferably 3 hours. Following treatment of 3C6 cells with a drug that stimulates T cell activation, a ratio of 59 kDa lck to total lck in stimulated drug treated 3C6 cells at least 20 percent, and preferably 60% higher than the ratio of 59 kDa lck to total lck in stimulated untreated cells is observed.

EXAMPLE VII

Testing ability of compounds to inhibit T cell activation.

Methods

P13.9 cells are intracellularly loaded with magnetic beads and used as antigen presenting cells (APCs) in the presence of 10-100 μM purified PCC(81-104) peptide for various amounts of time to stimulate 3C6 cells. The 3C6 cells are either not treated or previously, concurrently, or soon to be treated with a compound being tested for an ability to inhibit T cell activation. T cells are then isolated from the co-culture by magnetic depletion of the fibroblasts and immediately lysed. The lysate (6×10^5 T cell equivalents) is SDS-PAGE resolved and immunoblotted with an anti-lck antibody. Relative amounts of lck were quantitated on a densitometer.

Results

Peptide plus P13.9 stimulated untreated 3C6 cells are found to produce a higher ratio of 59 kDa lck to total lck than unstimulated untreated 3C6 cells following stimulation for 3 to 24 hours. Following treatment of 3C6 cells with a drug that inhibits T cell activation, a ratio of 59 kDa lck to total lck in stimulated drug treated 3C6 cells at least 50% lower than the ratio of 59 kDa lck to total lck in stimulated is observed.

EXAMPLE VIII

Serine phosphorylation-mediated redistribution and degradation of p56 lck.

Methods

Recombinant DNA technology was employed (using standard techniques described in, for example, Ausubel *et al.*, Current Protocols in Molecular Biology, John Wiley & Sons, New York, NY, 1994; or in Sambrook, Fritsch and Maniatis, Molecular Cloning: A Laboratory Manual (2d ed.), CSH Press, 1989) to generate two fusion proteins. The first fusion protein was a dimeric fusion, "lck-GFP." This fusion protein was generated by cloning the murine lck-encoding cDNA (Marth *et al.*, Cell 43: 393-404, 1985; Marth *et al.*, Proc. Natl. Acad. Sci. U.S.A. 83: 7400-7404, 1986; GenBank Accession No.: M12056), into one of the pEGFP vectors commercially available from Clontech, with the carboxy terminus of lck modified to allow fusion of the lck open reading frame with the green fluorescent protein (GFP) in the pEGFP vector. The second fusion protein, "CD4-lck-GFP," was a trimeric fusion of the CD4 extracellular domain fused to the amino terminus of lck (Xu and Littman, Cell 74(4): 633-643,1993), with GFP fused, as before, to the carboxy terminus of lck. These chimera-encoding expression vectors were then transiently transfected into BHK-21 cells (baby hamster kidney cells) using the FuGENE reagent (Boehringer-Mannheim; Catalog No. 1814443). In addition, stable transfections with these constructs are generated in the DAP.3 cell line (murine fibroblasts), which are selected in 1 mg/ml G418 (Boehringer-Mannheim).

Results

Following transfection of BHK-21 cells, lck-GFP (which could be easily visualized with the fluorescent marker, GFP), was found to localize primarily at the cell membrane as well as at an intracellular organelle. CD4-lck-GFP, on the other hand, formed a tight "cap" at the cell membrane. Serine phosphorylation was induced upon each chimera by phorbol-ester (PMA)-mediated activation of protein kinase C (PKC). Following 0, 15, 30, 60, or 90 minutes of stimulation with 12.5 μg/ml PMA, cells transfected with the pEGFP vector only (control), lck-GFP (lck:pEGFP), or CD4-

lck-GFP (CD4:lck:pEGFP) were photographed. Following stimulation, each chimera was found to rapidly redistribute within the cell and undergo degradation (Fig. 13). Preliminary biochemical analysis has shown that there is significant degradation of the lck-GFP chimera upon serine phosphorylation by PKC activation. Further biochemical analysis is carried out using the stably transfected DAP.3 cells.

EXAMPLE IX

Mutation of Serine-dileucine motifs.

Methods

After studying the sequences of various src-family tyrosine kinases, I observed that several, including lck, have at least one motif of Ser-X_{8-9}-Leu-Leu (SEQ ID NOs: 27 and 28), as shown on Fig. 14. I predict that these serine-dileucine motifs are essential in serine-phosphorylation mediated degradation of lck (as well as other src-family members). In murine lck, three motifs exist: 5' SRKDAERQLLAP 3' (SEQ ID NO: 2), found in the SH2 domain; 5' SGIKLNVNKLLDM 3' (SEQ ID NO: 3), found in the amino terminal half of the kinase domain; and 5' SDVWSFGILLTE 3' (SEQ ID NO: 4), found at the carboxy-terminus of the kinase domain. Using the known cDNA sequence of murine lck (Marth *et al.*, Cell 43: 393-404, 1985; Marth *et al.*, Proc. Natl. Acad. Sci. U.S.A. 83: 7400-7404, 1986; GenBank Accession No.: M12056), I will mutate each of the 5' serines of these motifs to alanine, and express these constructs in transfected BHK-21 and/or DAP.3 cells. I will also combine the mutations, such that I will eventually create and express a murine lck having alanine residues that replace the 5' serines in all three of SEQ ID NOs: 2-4). In addition to mutating the serine residues, I will mutate the di-leucine residues in the above motifs to di-alanine residues in the same mutated lck-encoding cDNAs, and express these in transfected BHK-21 and/or DAP.3 cells.

Results

Transfected cells will be analyzed for lck localization and degradation before and after stimulation with phorbol ester, as described above. Inhibition of differentiation and apoptotic death in stable transfectants will also be analyzed.

Other Embodiments

All publications mentioned in this specification are herein incorporated by reference to the same extent as if each independent publication was specifically and individually indicated to be incorporated by reference.

While the invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications and this application is intended to cover any variations, uses, or adaptations of the invention following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth, and as follows in the scope of the appended claims.

What is claimed is:

SEQUENCE LISTING

(1) GENERAL INFORMATION

(i) APPLICANT: The John P. Robarts Research Institute

(ii) TITLE OF THE INVENTION: LCK SERINE PHOSPHORYLATION ASSAY FOR T CELL ACTIVATION AND ACTIVATION-INDUCED CELL DEATH

(iii) NUMBER OF SEQUENCES: 25

(iv) CORRESPONDENCE ADDRESS:
(A) ADDRESSEE: Clark & Elbing LLP
(B) STREET: 176 Federal Street
(C) CITY: Boston
(D) STATE: MA
(E) COUNTRY: USA
(F) ZIP: 02110

(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Diskette
(B) COMPUTER: IBM Compatible
(C) OPERATING SYSTEM: DOS
(D) SOFTWARE: FastSEQ for Windows Version 2.0

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE: 10-APR-1998
(C) CLASSIFICATION:

(vii) PRIOR APPLICATION DATA:
(A) APPLICATION NUMBER: 60/041,878
(B) FILING DATE: 11-APR-1997

(viii) ATTORNEY/AGENT INFORMATION:
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(C) REFERENCE/DOCKET NUMBER: 50068/002WO2

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(A) TELEPHONE: 617-428-0200
(B) TELEFAX: 617-428-7045
(C) TELEX:

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 24 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ile Phe Ala Gly Ile Lys Lys Lys Ala Glu Arg Ala Asp Leu Ile Ala
 1               5                  10                  15
Tyr Leu Lys Gln Ala Thr Ala Lys
            20
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
Ser Gly Ile Lys Leu Asn Val Asn Lys Leu Leu Asp Met
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 12 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 12 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

```
Ser Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 11 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```
Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```
Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

```
Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

```
Arg Lys Asp Ala Glu Arg Gln Leu Leu Ala Pro
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 11 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Gly Ile Lys Leu Asn Lys Leu Leu Asp Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 13 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Ser Gly Ile Lys Leu Asn Val Asn Lys Leu Leu Asp Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Gly Ile Lys Leu Asn Lys Leu Leu Asp Met
1 5 10

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                   10
```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
1 5 10

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

```
Ser Asp Val Trp Ser Phe Gly Ile Leu Leu Thr Glu
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 10 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

(A) NAME/KEY: Other
(B) LOCATION: 1...10
(D) OTHER INFORMATION: Xaa is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

```
Ser Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Leu
 1               5                  10
```

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:
  (A) LENGTH: 11 amino acids
  (B) TYPE: amino acid
  (C) STRANDEDNESS: single
  (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide
(ix) FEATURE:

  (A) NAME/KEY: Other
  (B) LOCATION: 1...11
  (D) OTHER INFORMATION: Xaa is any amino acid

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

```
Ser Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Leu Leu
 1               5                   10
```

Claims

1. A method for detecting the level of T cell activation, said method comprising the steps of:

(a) providing a first sample comprising a T cell;

(b) measuring the amount of the serine phosphorylated lck in said first sample; and

(c) measuring the amount of total lck in said first sample, wherein an increase in the ratio of serine phosphorylated lck to total lck relative to the ratio of serine phosphorylated lck to total lck in a second sample consisting essentially of unstimulated T cells indicates increased T cell activation in said first sample, and wherein a decrease in said ratio of serine phosphorylated lck to total lck relative to the ratio of serine phosphorylated lck to total lck in said second sample indicates increased T cell activation-induced cell death in said first sample.

2. The method of claim 1, wherein said first sample and said second sample are from two different individuals.

3. The method of claim 1, wherein said first sample and said second sample are from the same individual, and said second sample is collected from said individual prior to the collection of said first sample.

4. The method of claim 1, wherein said T cell of said first sample and said unstimulated T cells of said second sample are $CD4^{+}$ helper T cells.

5. The method of claim 1, wherein said T cell of said first sample is a stimulated T cell.

6. The method of claim 5, wherein said stimulated T cell is stimulated with a peptide-MHC complex.

7. The method of claim 5, wherein said stimulated T cell is stimulated for at least 1 hour.

8. The method of claim 5, wherein said stimulated T cell is stimulated optimally for 3 hours.

9. The method of claim 5, wherein said stimulated T cell is stimulated for less than 24 hours.

10. The method of claim 6, wherein said peptide-MHC complex is expressed on an antigen-presenting cell.

11. The method of claim 1, wherein said measuring is conducted in a cell-free system.

12. The method of claim 10, wherein said lck is a recombinant protein.

13. The method of claim 10, wherein said T cell activation is initiated by components which mimic the T cell receptor.

14. The method of claim 1, wherein said detecting is for identifying a compound that modulates T cell activation.

15. A method for detecting a compound that modulates T cell activation, said

method comprising the steps of:

(a) providing a first sample comprising a T cell;

(b) contacting said first sample with a compound;

(c) measuring the amount of the serine phosphorylated lck in said compound-contacted first sample; and

(d) measuring the amount of total lck in said compound-contacted first sample, wherein a change in the ratio of serine phosphorylated lck to total lck in said first sample, relative to the serine phosphorylated lck to total lck ratio in a second sample comprising a T cell, said second sample not contacted with said compound, indicates said compound modulates T cell activation.

16. The method of claim 15, wherein said T cell of said first sample and said second sample is a stimulated T cell.

17. The method of claim 15, wherein said compound that modulates T cell activation is a compound that increases T cell activation, and said change is at least a 20% increase as compared to said second sample.

18. The method of claim 17, wherein said change is optimally a 60% increase as compared to said second sample.

19. The method of claim 16, wherein said compound that modulates T cell activation is a compound that increases T cell activation-induced cell death and said change is at least a 50% decrease as compared to said second sample.

20. A kit for assaying T cell activation, said kit comprising:

a) an antibody that specifically binds lck; and

b) means for determining the apparent molecular mass of said lck.

21. A src-family tyrosine kinase polypeptide having a mutation that reduces serine phosphorylation-mediated degradation, said mutation being a mutation that results in a serine to alanine change in the polypeptide, said mutation being in the serine residue located at the amino terminus of a sequence, said sequence selected from a group of sequences consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, and SEQ ID NO: 25.

22. The polypeptide of claim 21, wherein said polypeptide is lck.

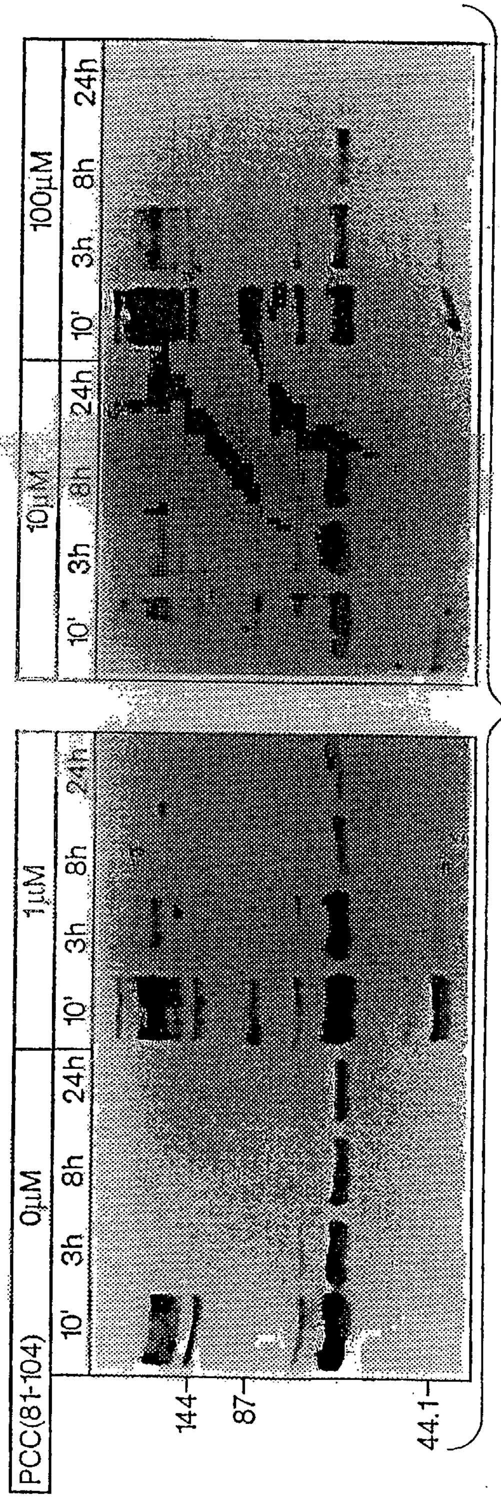
23. A src-family tyrosine kinase polypeptide having a mutation that reduces serine phosphorylation-mediated degradation, said mutation being a mutation that results in a leucine-leucine to alanine-alanine change in the polypeptide, said mutation being in the leucine-leucine residues located within a sequence, said sequence selected from a group of sequences consisting of SEQ ID NO: 2, SEQ ID NO: 3, SEQ ID NO: 4, SEQ ID NO: 5, SEQ ID NO: 6, SEQ ID NO: 7, SEQ ID NO: 8, SEQ ID NO: 9, SEQ ID NO: 10, SEQ ID NO: 11, SEQ ID NO: 12, SEQ ID NO: 13, SEQ ID NO: 14, SEQ ID NO: 15, SEQ ID NO: 16, SEQ ID NO: 17, SEQ ID NO: 18, SEQ ID NO: 19, SEQ ID NO: 20, SEQ ID NO: 21, SEQ ID NO: 22, SEQ ID NO: 23, SEQ ID NO: 24, and SEQ ID NO: 25.

24. The polypeptide of claim 23, wherein said polypeptide is lck.

25. A method of generating a src-family tyrosine kinase polypeptide that has a reduced level of serine phosphorylation-mediated degradation, said method comprising the steps of:

(a) identifying SEQ ID NO: 27 or SEQ ID NO: 28 in said polypeptide; and

(b) mutating the 5' serine residue in said SEQ ID NO: 27 or said SEQ ID NO: 28 to alanine, whereby said mutation reduces the level of said serine phosphorylation-mediated degradation in said polypeptide.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1μM
10μM
100μM
10'
3h
8h
24h
144
87
44.1

Fig. 1

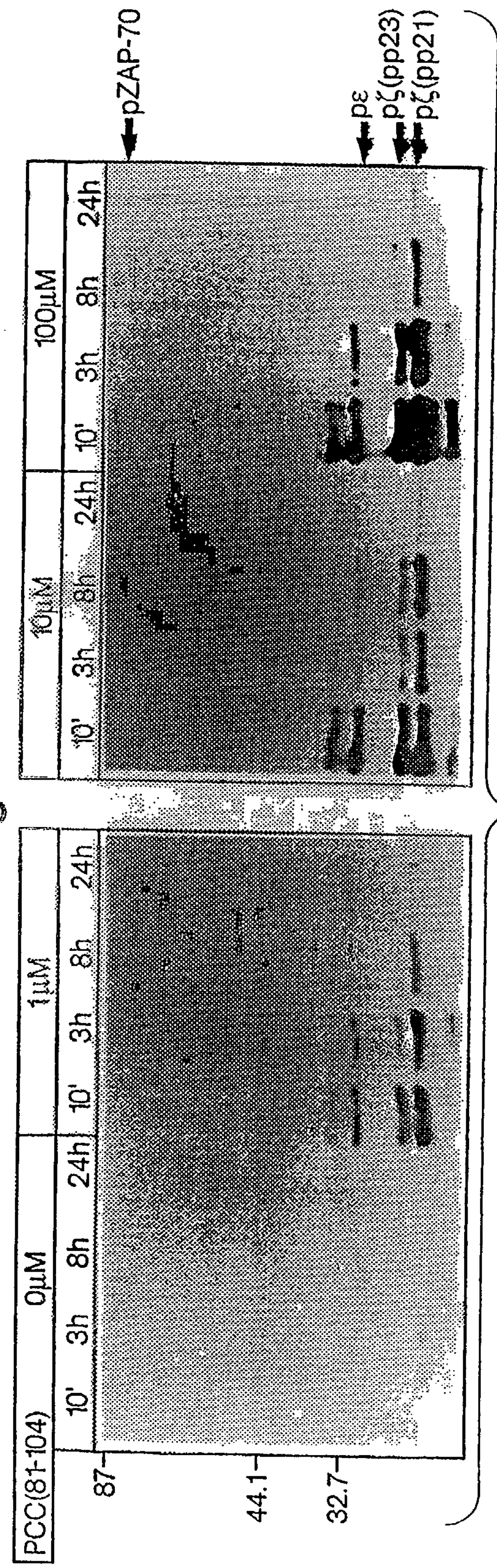
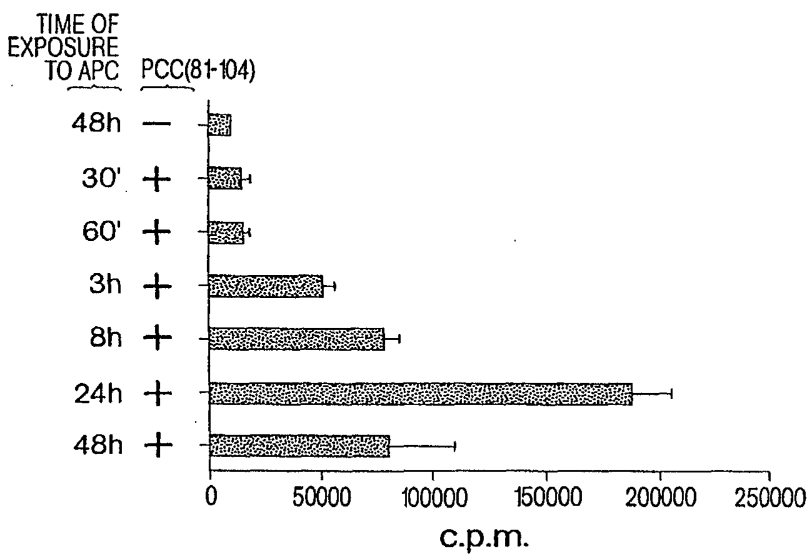

PCC(81-104)
0μM
1μM
10μM
100μM
10'
3h
8h
24h
87
44.1
32.7
pZAP-70
pε
pζ(pp23)
pζ(pp21)

Fig. 2



TIME OF EXPOSURE TO APC
PCC(81-104)
48h —
30' +
60' +
3h +
8h +
24h +
48h +
0
50000
100000
150000
200000
250000
c.p.m.

Fig. 3

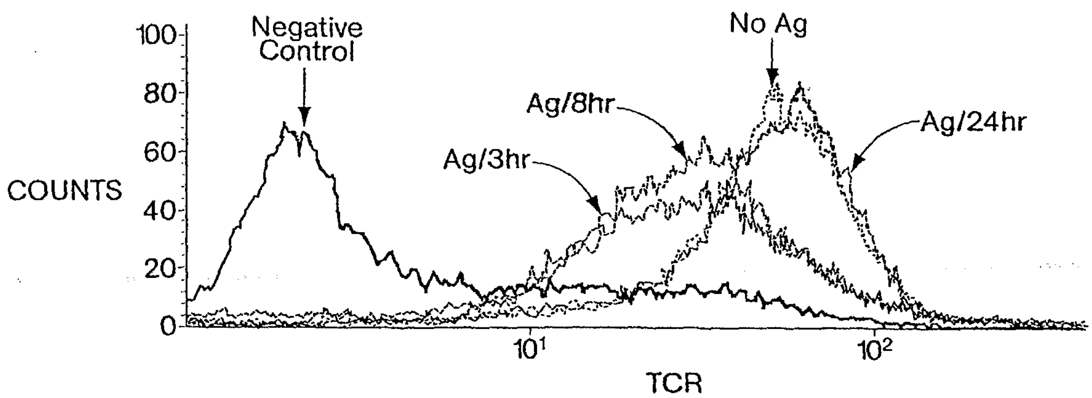


Fig. 4

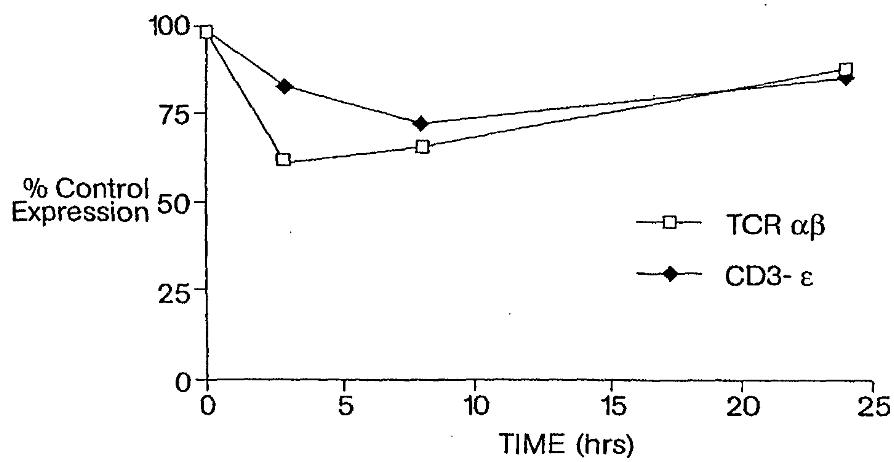


Fig. 5

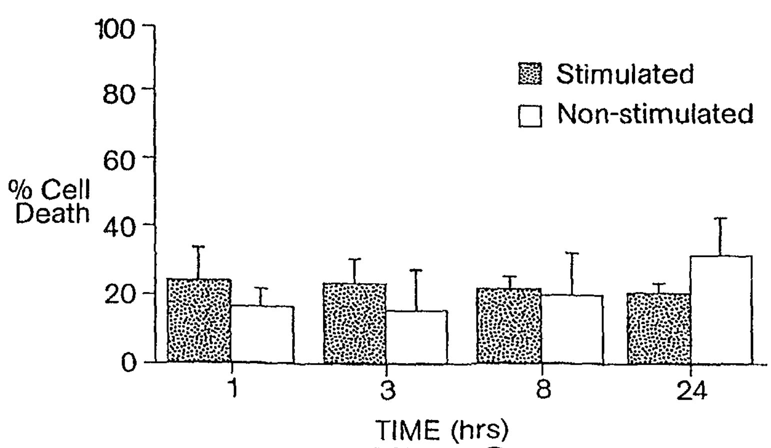
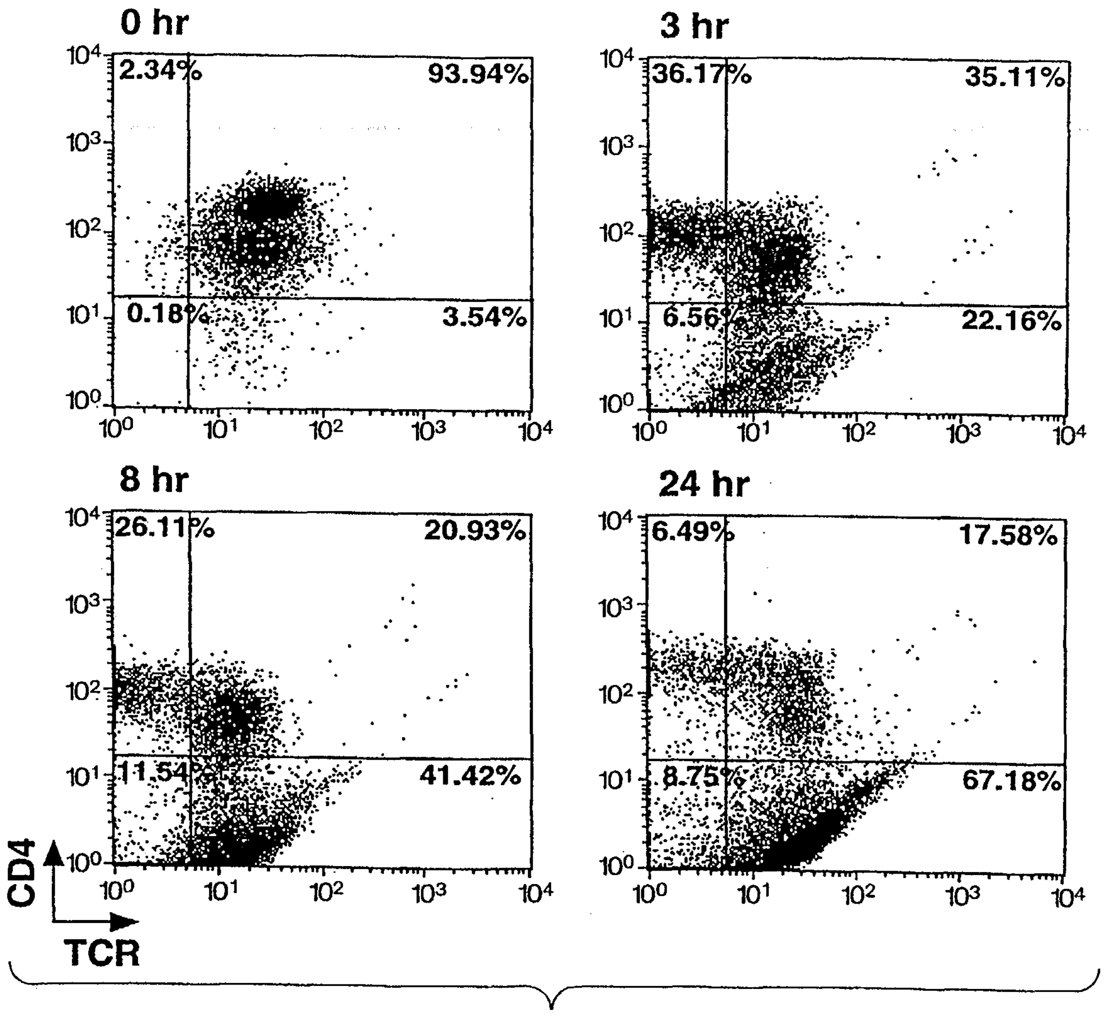


Fig. 6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3.54%
3 hr
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35.11%
6.56%
22.16%
8 hr
26.11%
20.93%
11.54%
41.42%
24 hr
6.49%
17.58%
8.75%
67.18%
CD4
TCR

Fig. 7

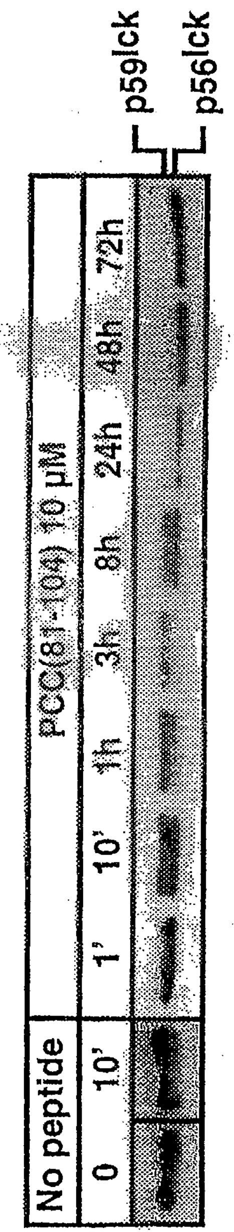


Fig. 8

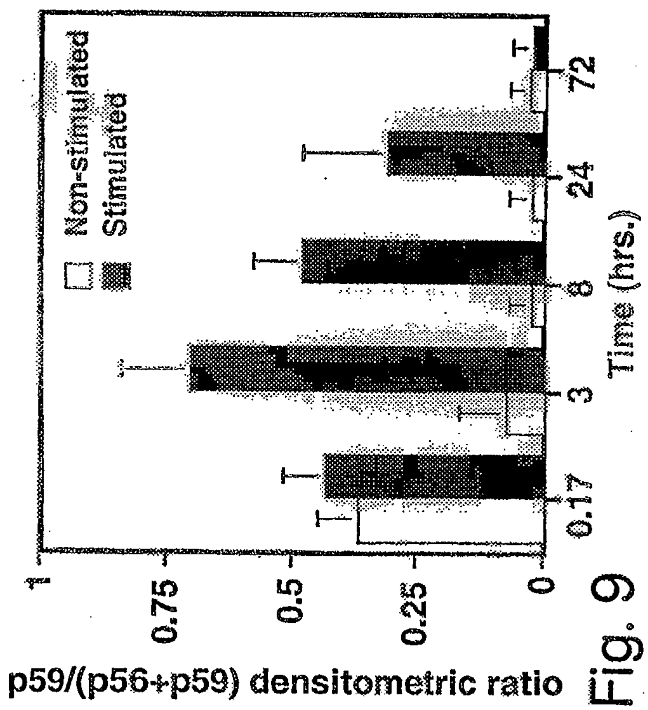


Fig. 9

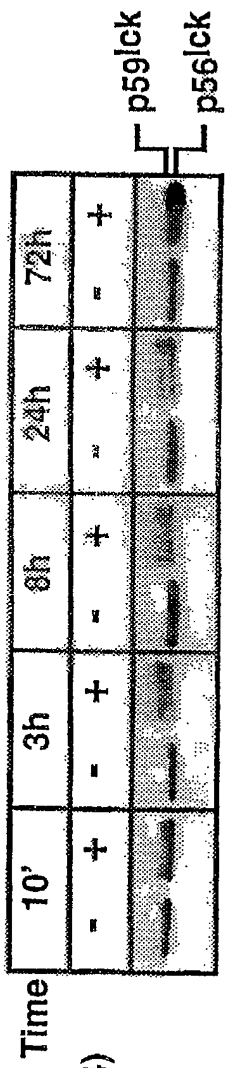


Fig. 10

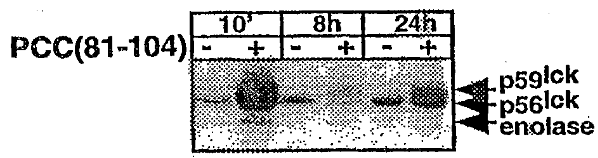

10'
8h
24h
PCC(81-104)
- + - + - +
p59lck
p56lck
enolase

Fig. 11

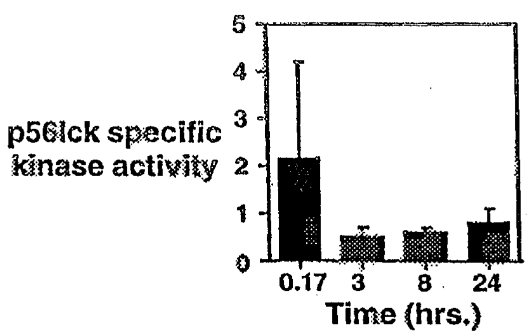

p56lck specific kinase activity
5
4
3
2
1
0
0.17
3
8
24
Time (hrs.)

Fig. 12

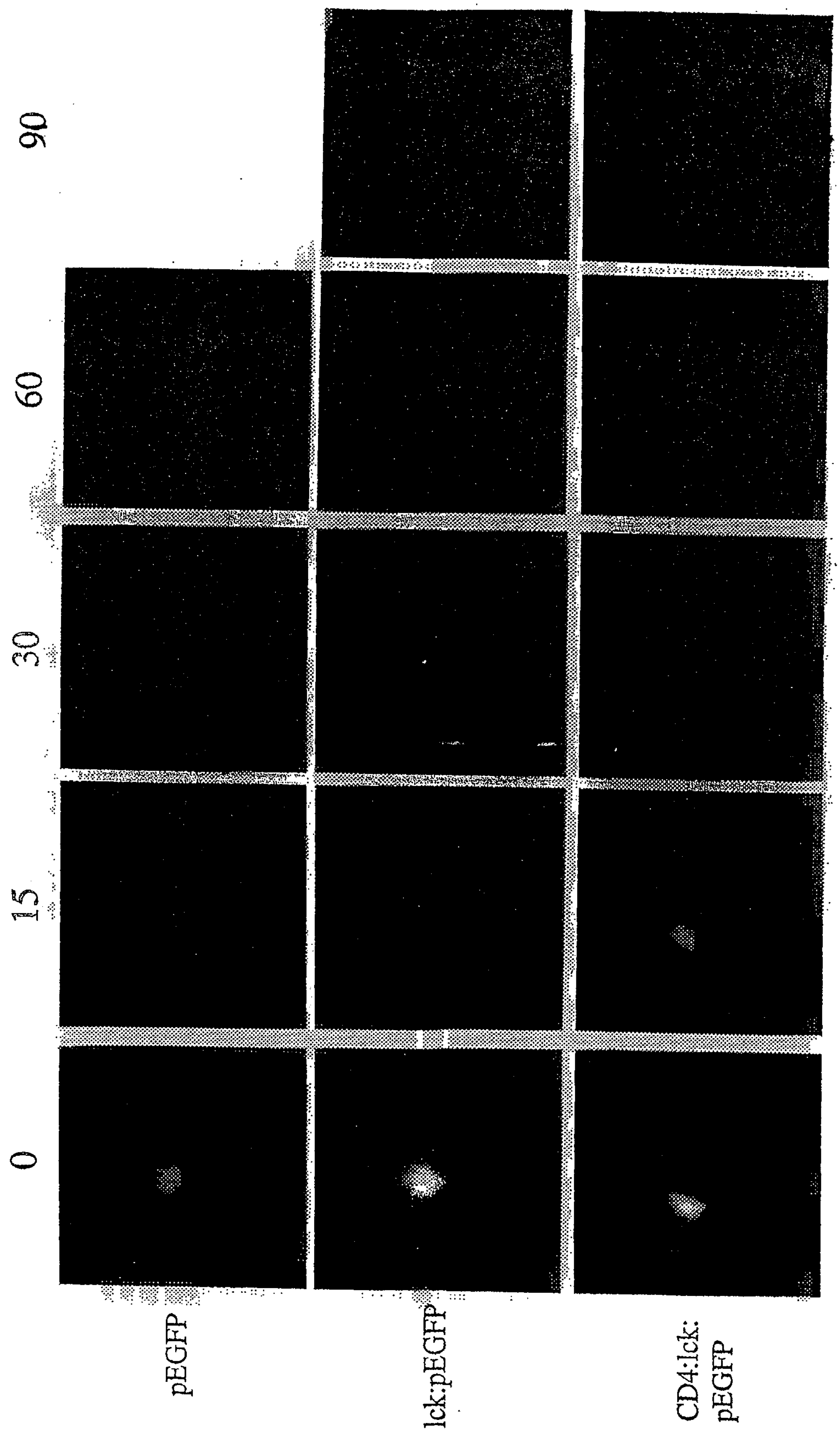
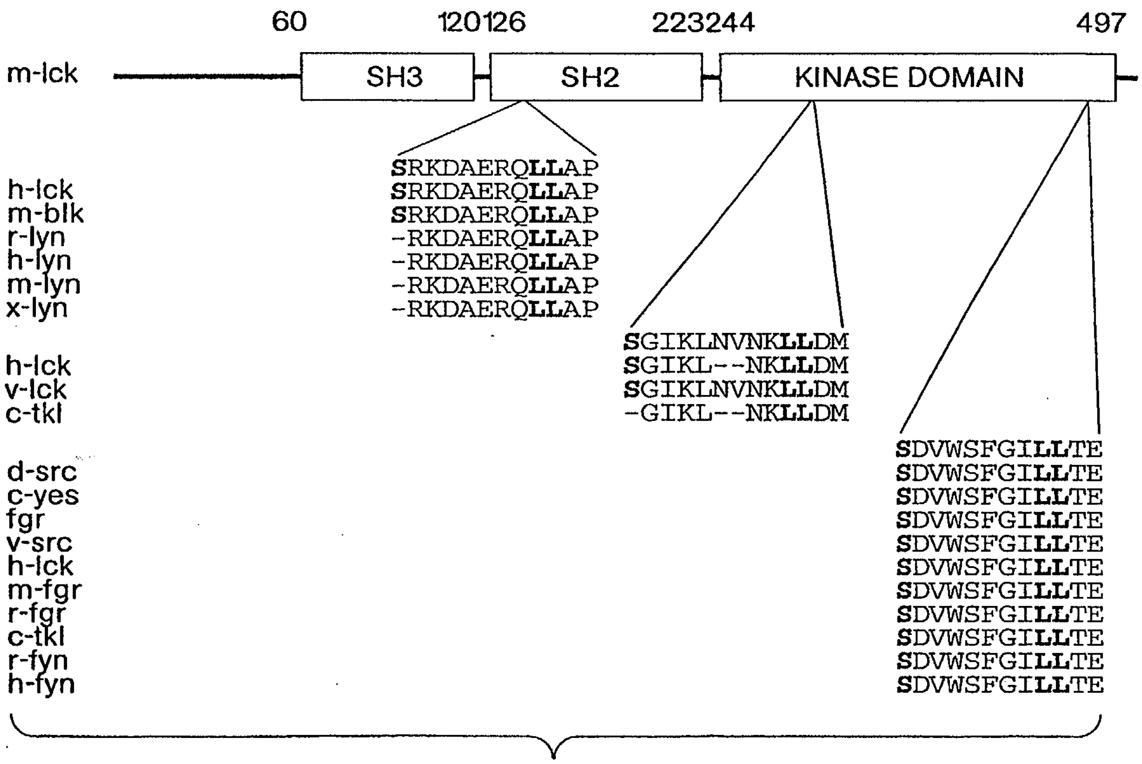


Fig. 13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120126
223244
497
m-lck
SH3
SH2
KINASE DOMAIN
SRKDAERQLLAP
h-lck SRKDAERQLLAP
m-blk SRKDAERQLLAP
r-lyn -RKDAERQLLAP
h-lyn -RKDAERQLLAP
m-lyn -RKDAERQLLAP
x-lyn -RKDAERQLLAP
SGIKLNVNKLLDM
h-lck SGIKL--NKLLDM
v-lck SGIKLNVNKLLDM
c-tkl -GIKL--NKLLDM
SDVWSFGILLTE
d-src SDVWSFGILLTE
c-yes SDVWSFGILLTE
fgr SDVWSFGILLTE
v-src SDVWSFGILLTE
h-lck SDVWSFGILLTE
m-fgr SDVWSFGILLTE
r-fgr SDVWSFGILLTE
c-tkl SDVWSFGILLTE
r-fyn SDVWSFGILLTE
h-fyn SDVWSFGILLTE

Fig. 14

INTERNATIONAL SEARCH REPORT

Internatio... Application No
PCT/IB 98/00801

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 G01N33/573 C12N9/12

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 G01N C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PATHAN, N. I. ET AL.,: "The protein kinase lck associates with and is phosphorylated by cdc2" THE JOURNAL OF BIOLOGICAL CHEMISTRY, vol. 271, no. 44, 1 November 1996, pages 27517-27523, XP002079585 see the whole document see page 27521, right-hand column, paragraph 2 see page 27517, right-hand column, line 5 - line 13 ---	1-20

-/--

[X] Further documents are listed in the continuation of box C.

[ ] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
5 October 1998	22.01.99

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Hoekstra, S

INTERNATIONAL SEARCH REPORT

International Application No
PCT/IB 98/00801

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	HORAK, I.D. ET AL.: "T.lymphocyte interleukin-2 dependent tyrosine protein kinase signal transduction involves the activation of p56lck" PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA, vol. 88, March 1991, WASHINGTON US, pages 1996-2000, XP002079586 see the whole document ---	1-20
X	MARTH, J.D. ET AL.: "Lymphocyte activation provokes modiication of a lymphocyte-specific proetin tyrosine kinase (p56lck)" JOURNAL OF IMMUNOLOGY, vol. 142, no. 7, 1 April 1989, BALTIMORE US, pages 2430-2437, XP002079587 see abstract ---	1-20
A	GOLD, M.R.: "Activation and serine phosphorylation of the p56lck protein tyrosine kinase in response to antigen receptor cross-linking in B lymphocytes" THE JOURNAL OF IMMUNOLOGY, vol. 153, no. 6, 15 September 1994, pages 2369-2380, XP002079588 see the whole document ---	1-20
A	REPKE, H. ET AL.: "ganglioside-induced CD4 endocytosis occurs independent of serine phosphorylation and is accompanied by dissociation of p56lck" THE JOURNAL OF IMMUNOLOGY, vol. 149, no. 8, 15 October 1992, pages 2585-2591, XP002079589 see the whole document ---	1-20
T	LEE, J.E. ET AL.: "Inactivation of lck and loss of TCR-mediated signalling upon persistent engagment with complexes of peptide:MHC molecules." THE JOURNAL OF IMMUNOLOGY, vol. 159, no. 1, 1 July 1997, pages 61-69, XP002079590 see the whole document -----	1-20

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB 98/00801

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This International Search Report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the International Application that do not comply with the prescribed requirements to such an extent that no meaningful International Search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

see additional sheet

1. ☐ As all required additional search fees were timely paid by the applicant, this International Search Report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this International Search Report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☒ No required additional search fees were timely paid by the applicant. Consequently, this International Search Report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

   1-20

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

FURTHER INFORMATION CONTINUED FROM PCT/ISA/ 210

1. Claims: 1-20

Assay for detecting level of T-Cell activation based on measuring serine phosphorylated lck and comparing with total lck as a measure for T-cell activation, use of this assay to identify compounds influencing the assay result and kits therefor.

2. Claims: 21-25

Mutated src-family tyrosine kinase polypeptides that are less sensitive to serine phosphorylation mediated degradation.